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# Dendritic cells used in anti-HIV immunotherapy showed different modulation in anti-HIV genes expression: New concept for the improvement of patients' selection criteria

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**Abstract**

*Objective*: As the host's genetics influences immune response to HIV-1 and progression to AIDS, similar genetic factors could affect response to immunotherapy. Differential genes expression was evaluated in clinical trial, aimed at identifying a potential predictive marker for DC quality and, finally, for therapy responsiveness.

*Methods and Results*: DC used for immunotherapy revealed a clear difference of anti-HIV genes signature, so we classified DC into two groups (A-DC and B-DC). In A-DC a limited number of genes, including inflammasome-related genes (*IL1B, IL18*), were modulated during monocytes-to-DC differentiation. A larger subset of anti-HIV genes (restriction factors, co-factors, apoptotic factors) was modulated in B-DC. This more "activated/exhausted" expression profile of B-DC apparently resulted from a more activated monocyte precursor.

*Conclusions*: These results suggest that the actual selection of $HIV^+$ individual for immunotherapy, based on clinical features, did not ensure the same DC product, and that less "activated/exhausted" DC could positively affect the outcome of immunotherapy.



*Keywords:* HIV-1; Immune-therapy; Dendritic cells; Genomic profile

## 1. Introduction

Dendritic cells (DC)-based immune-therapy (commonly called "therapeutic vaccine") has been reported as an interesting approach to induce a control of plasma viral load (PVL) in HIV positive ($HIV^+$) patients as well as an important tool for deeper investigating the correlation of protection against HIV infection in these patients.

Since the first published results [1–3], it appeared that not all the immunized patients uniformly respond to the treatment,

*Abbreviations:* DC, (monocyte derived) dendritic cells; $HIV^+$, seropositive individuals; PBMC, peripheral blood mononuclear cells; PVL, plasma viral load.

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opening a plethora of question about the characterization of factors that might affect the out-come of immunization, and the definition of guide lines to appropriately choose individuals with greatest chance to effectively respond to immune-therapy.

Genetics screening of HIV$^+$ patients submitted to the first-phase clinical trial of a French-Brazilian DC-based vaccine [3] evidenced that polymorphisms in *MBL2*, *NOS1*, *PARD3B* and *CNOT1* genes were associated with a weak or transient response (not significantly diminished PVL) observed in half of 18 treated subjects [4–6].

The profile of "weak/transient" or "good" responder may also depend on several factors, other than host genome, such as the quality of DC obtained *in vitro* from patient's peripheral blood monocytes, and the ability of patient's immune system to be activated by the *in vitro* manipulated DC. Considering that immune response of HIV$^+$ individuals is greatly impaired by HIV-1 infection itself, questions about the responsiveness to a DC-based vaccine are strictly correlated with the ability of each individual to counteract the infection.

In this context we considered that exploring DC gene expression profile may be helpful for understanding genes or pathways linking DC biology and a good response to immunotherapy, and finally for the selection of individuals that can be benefit from this type of intervention.

Nowadays other 20 Brazilian HIV$^+$ patients were enrolled in the second-phase of Lu et al. clinical trial [3], and biologic samples are becoming available for novel investigation.

All this considered and taken into account that the intrinsic ability of each HIV$^+$ individual to counteract HIV-1 results in a different rate of immune cells activation [7,8] and consequently in a different capacity of HIV$^+$ to be responsive toward exogenous stimulation (i.e.: immunotherapy), we decided to study differential expression of genes involved in host anti-HIV response in available cells from 6 HIV$^+$ patients included in the phase-I/II clinical trial. To determine whether this expression profile is different among vaccinated individuals and if an alteration of this profile could eventually be prejudicial to immunotherapy, HIV restriction genes expression was evaluated in different steps of monocytes-to-DC preparation according to immunotherapy protocol [3] and correlated with DC characteristics and functions, HIV restriction factors genetics, and with clinical trial results.

## 2. Material and methods

### 2.1. Patients

Six HIV-1 positive Brazilian individuals were selected within the subjects submitted to anti-HIV immunotherapy clinical trial at the Laboratory of Medical Investigation/LIM-56 (Faculty of Medicine, University of Sao Paulo, Brazil) due to the availability of biologic material. All individuals were males, adults (31.3 ± 7.6 years), classified as European-derived according to an appropriate questionnaire [9,10], proceeding from Sao Paulo city geographical area. They are seropositive for at least 5 years, naïve for antiretroviral therapy and without clinical AIDS or other chronic diseases, with blood CD4$^+$ cells count >500 cells/ml, and PVL >3 log (1000 RNA copies/ml). Patients' main characteristics are summarized in Table 1. Detailed PVL and CD4$^+$ data collected during treatment follow-up are reported in Supplementary Table 1. Written informed consent was obtained according to the protocol of "Hospital das Clinicas" Ethical Committee (CAPPesq) (number 0791/09, 04 November 2009).

### 2.2. Monocyte-derived dendritic cells

Monocyte-derived dendritic cells (DC) were obtained and stimulated according to the protocol used in anti-HIV immunotherapy by Lu et al. [3]. Briefly, peripheral blood mononuclear cells (PBMC), obtained by centrifugation over Ficoll-Paque gradient, were distributed in 24-wells plates at $5 \times 10^6$/well, and monocytes isolated by adherence and cultured in AIM-V medium (*Gibco, Life Technologies*) containing 50 ng/mL GM-CSF (*Cell Genix*) and 50 ng/mL IL-4 (*Cell Genix*). Non-adherent PBMC were used for co-culture assays. On day 5, immature DC (iDC) were pulsed with alditrithiol-2 inactivated HIV-1 ($1 \times 10^9$ viral particles/$30 \times 10^6$ cells) for 4 h (4 h-DC), then cells were washed and DC "maturation" cocktail (50 ng/mL IL-4, 50 ng/mL GM-CSF, 50 ng/mL tumour necrosis factor (TNF), 10 ng/mL IL-1β, 100 ng/mL IL-6) (*Cell Genix)* was added for further 10 (14 h-DC), 20 (24 h-DC) or 44 h (48 h-DC). 48 h-DC represent the mature DC and the final product of the manipulation. iDC and 48 h-DC were analysed for dendritic cell differentiation and activation markers by flow-cytometry. Viability of 48 h-DC was evaluated. Cells were lysed for mRNA isolation and for gene expression analysis at all the above-mentioned time-points (monocytes, iDC, 4 h-, 14 h-, 24 h-, 48 h-DC).

### 2.3. Virus isolation and expansion

Virus isolation and expansion were performed according to WHO–UNAIDS Guidelines [11] with minor modifications [3]. Viral inactivation was made with Aldrithiol™-2 as described elsewhere [12].

### 2.4. RNA isolation and RT$^2$ profiler PCR array

Total RNA was isolated using the RNAeasy mini kit (*Qiagen*) and quantified using Nanodrop N-1000 (*Agilent*

Table 1
**Main characteristics of HIV$^+$ subjects selected for the study.**

| ID | Age/Sex | PVL (copies/ml; $\log_{10}$) | CD4$^+$ count (cells/μl) | CD8$^+$ count (cells/μl) |
|---|---|---|---|---|
| P1 | 40/M | 21,420; 4.3 | 566 | 776 |
| P2 | 35/M | 38,295; 4.6 | 500 | 1100 |
| P3 | 27/M | 5003; 3.7 | 500 | 1139 |
| P4 | 24/M | 24,530; 4.4 | 569 | 868 |
| P5 | 23/M | 1342; 3.1 | 684 | 822 |
| P6 | 39/M | 1237; 3.1 | 500 | 1371 |

Abbreviations: M = male; PVL = plasma viral load; CD$^4$+ = CD$^4$+ T lymphocytes, CD8$^+$ = CD8$^+$ T lymphocytes.

*Biosystems*). 0.5−1 μg total RNA was retro-transcribed with the RT$^2$ PreAMP cDNA Synthesis Kit (Qiagen). Samples were analysed for expression of 84 genes involved in host genome anti-HIV response by RT$^2$ Profiler PCR "Host Genome Anti-HIV" Array (PAHS-051Y, Qiagen). Real-time PCR detection was performed on ABI 7300 Real-Time PCR System (Applied Biosystems). RT$^2$ Profiler PCR Array data were analysed using the comparative Ct method as proposed by Schmittgen & Livak [13]. This method calculates differences in Ct data, normalized for house-keeping genes (ΔCt), between two samples as fold change/FC. Average Ct of 4 housekeeping genes (β-actin/*ACTB*, β-2-microglobulin/*B2M*, glyceraldehyde-3-phosphate dehydrogenase/*GAPDH*, hypoxanthine phosphoribosyl-transferase 1/*HPRT1*) for each individual was used for normalization. Ct > 35 were excluded. Triplicates were used for each analysis. Genes with two missing values within the groups were excluded from analysis. Student's t-test was used to calculate two-tail, equal variance p-values in each triplicate. Quality controls confirmed the lack of DNA contamination and were successfully tested for RNA quality and PCR performance. Heatmaps and clustering were obtained with R-projects packages "gplots".

### 2.5. RT-PCR with Taqman assays

*NLRP3, CASP1* and *IL18* genes were amplified with specific TaqMan Gene Expression Assays (*Applied Biosystems*) using the ABI 7300 SDS platform (*Applied Biosystems*). *ACTB* was the housekeeping gene used for normalization. Relative quantitative expression was obtained using the comparative Ct method as proposed by Schmittgen & Livak [13]. Data was analysed by One-Way Anova and Bonferroni post-test in GraphPad Prism software.

### 2.6. Analysis of polymorphisms in HIV restriction factor genes

Twenty-two polymorphisms in 13 genes involved in HIV-1 host restriction genes (rs3736685 and rs2294367 in *APOBEC3G,* rs1719153 and rs1719134 in *CCL4,* rs2280789 and rs2107538 in *CCL5*, Δ32 in *CCR5,* rs11212495 and rs7103534 in *CUL5,* rs2234358 in *CXCR6,* rs10484554 and rs9264942 in *HLA-C,* rs2069709 in *IFNG,* rs11884476 in *PARD3B,* rs17762192 in *PROX1,* rs1801157 in *SDF-1,* rs16934386, rs10838525 and rs3740996 in *TRIM5*, rs3869068 and rs8321 in *ZNRD1*) were analysed in our patients. These polymorphisms were previously studied by our group in the context of phase I anti-HIV immunotherapy clinical trial [5]. Genotyping was performed using commercially available TaqMan assays (Applied Biosystems/AB) and ABI7500 Real-Time platform (AB). Allelic discrimination was performed using the SDS v1.4 Software (AB). *CCR5* Δ32 deletion was evaluated by PCR-RFLP.

### 2.7. Phenotypic analysis

Monocytes, DC and lymphocytes were analysed for common characterization markers by flow cytometry. CD14, HLA-DR, CD11c and CD86 surface markers were used for monocytes; CD11c, CD40, CD80, CD83, CD86, HLA-DR for DC; CD3, CD38 for lymphocytes. Nonspecific IgG1, IgG2a, a mixture of IgG1 and IgG2a were used as controls. All antibodies were from *BD Biosciences*. Analysis was performed on a FACSCalibur™ cell analyser (*BD Biosciences*) and FLOWJO software. Data were analysed by t test or ANOVA using GraphPad Prism software.

### 2.8. Viability assay

48 h-DC viability was evaluated by propidium iodide (PI) staining and flow cytometry, according to manufacturer instruction (*BD Biosciences*). Analysis was performed on a FACSCalibur™ cell analyser (*BD Biosciences*) and FLOWJO software. Data were analysed by t-test using GraphPad Prism software. Data were reported as percentage of PI negative cells.

### 2.9. DC-mediated T lymphocyte activation

Autologous T lymphocyte activation by DC was measured evaluating CD38 surface expression and IFN-γ production. Briefly, $2 \times 10^5$ autologous non-adherent PBMC were co-cultured with $0.4 \times 10^5$ DC per well in a 96-well plate for 96 h. CD38 surface expression was analysed as above-mentioned, whether IFN-γ production by intracellular staining. Briefly, 20 μg/mL BrefeldinA (Sigma-Aldrich) was added to block protein secretion for the last 4 h of the culture period. At the end of co-culture, cells were stained for surface marker CD3 (*BD Bioscience*), then permeabilized with BD Cytofix/Cytoperm solution (*BD Biosciences*) and stained for intracellular IFN-γ (*BD Biosciences*). Stimulation with Staphylococcal enterotoxin B (SEB) was used as positive control. Analysis was performed on a FACSCalibur™ cell analyser (*BD Biosciences*) and FLOWJO software. Data were analysed by t-test using GraphPad Prism software.

## 3. Results

Gene expression of monocytes-derived dendritic cells from 6 HIV$^+$ patients submitted to immunotherapy was examined comparing, in each individual, 5 differentiation's steps (iDC, 4 h-DC, 14 h-DC, 24 h-DC and 48 h-DC) versus monocytes.

Clustering analysis obtained for the 6 patients on all DC time-points showed the segregation of DC in two independent clusters according to the donor rather than to differentiation step or genes (group A: P1, P5 and P6; group B: P2, P3 and P4). DC from group A presented a general down-regulation of anti-HIV response genes, while an up-regulation was observed in DC from group B (Fig. 1), in an apparently uniform way along differentiation. This finding was intriguing because HIV$^+$ individuals selected for the clinical trial were clinically homogeneous (seropositive for at least 5 years, absence of anti-retroviral treatment, CD4$^+$ >500,000, PVL>3log; see Table 1), moreover the 6 studied patients are all males, with a similar age and race.

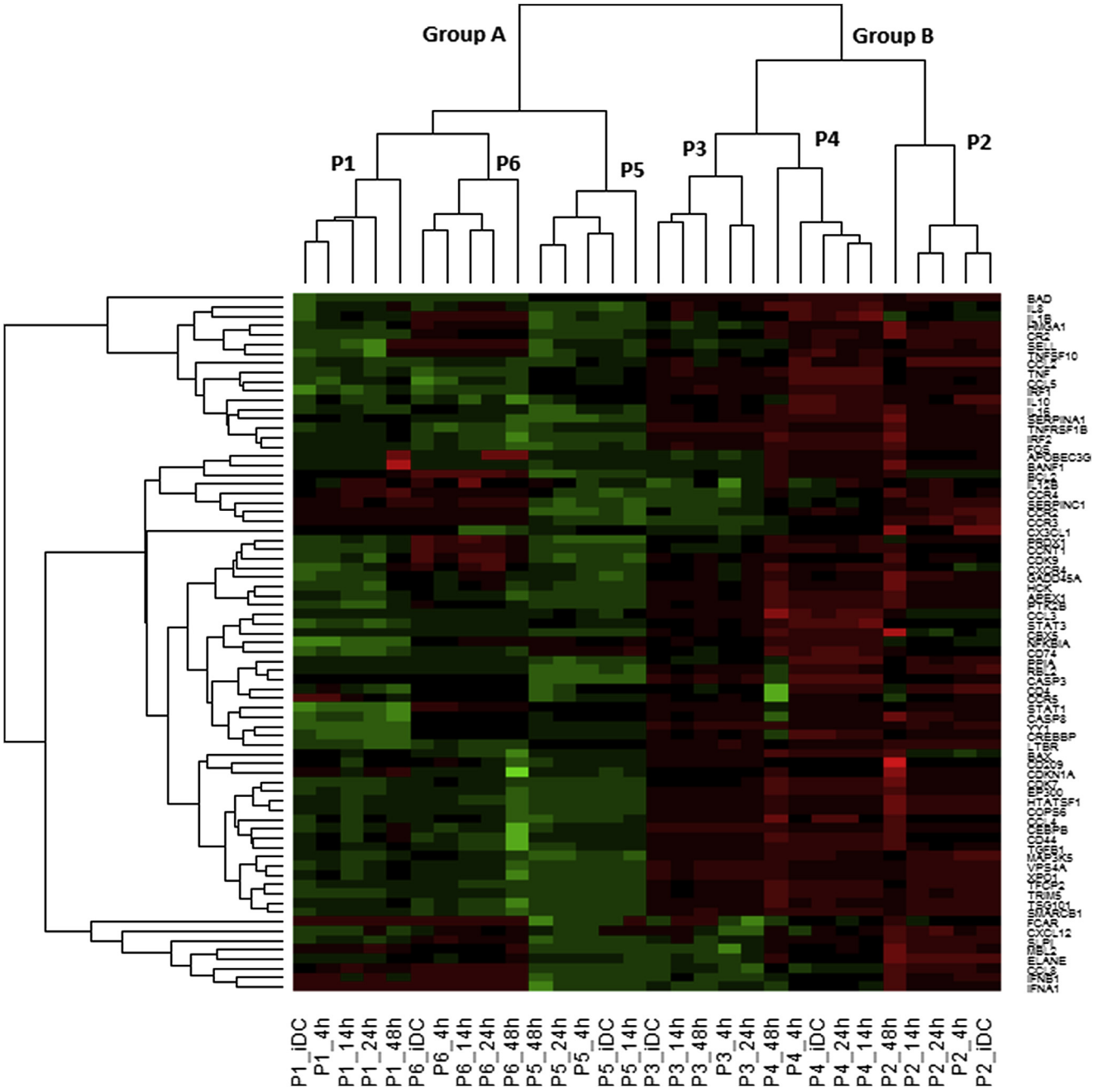


Fig. 1. **Clustering of genes differentially expressed during in vitro monocyte-to-DC differentiation**. Heatmap reports $log_2$FC values for 84 genes of Host anti-HIV response gene pathway array (Qiagen) in iDC, 4 h-DC, 14 h-DC, 24 h-DC and 48 h-DC compared with monocytes. The clustering is defined by the dendrogram on the top of the clustergram.

First we investigated the possible cause of this difference looking at the available clinical data. Correlation analysis between PVL, $CD4^+$ and $CD8^+$ T lymphocytes counts and gene expression did not evidence any association with expression profile in DC from A and B groups (data not shown). However, taking in account the limited size of studied individuals and the absence of clinical significant reduction of PVL (<1 log), we can observe a higher, even not statistically significant ($p > 0.05$), mean levels of T $CD4^+$ lymphocytes during the treatment in group A compared to B (Table 2, and Supplementary File 1).

Polymorphisms in HIV restriction factor genes (*APOBEC3G, CCL4, CCL3, CCL5, CCR5, CUL5, CXCL12, CXCR6, HLA-C, IFNG, PARD3B, PROX1, TRIM5, ZNRD1*) were previously evaluated in the context of immunotherapy [5] suggesting that, at least *PARD3B,* appeared to be associated to a "good" response in the phase-I clinical trial. So we considered whether HIV restriction factors could affect the immunotherapy out-come influencing DC biology in $HIV^+$ individuals. For this purpose, frequency of selected polymorphisms in *APOBEC3G, CCL4, CCL3, CCL5, CCR5, CUL5, CXCL12, CXCR6, HLA-C, IFNG, PARD3B, PROX1,*

Table 2
**Difference in plasma viral load (PVL) and T CD4$^+$ lymphocytes in HIV$^+$ patients after anti-HIV-1 immunotherapy**. Differences in log(PVL) and CD4$^+$ lymphocytes counts during 36-weeks follow-up were reported. Difference between group A and group B values was analysed by t test. P-values <0.05 were considered statistically significant.

| Follow-up (weeks) | 2 | 4 | 6 | 12 | 24 | 36 |
|---|---|---|---|---|---|---|
| **ΔPVL (log)** | | | | | | |
| Group A | 0.25 | 0.32 | 0.18 | 0.16 | 0.15 | 0.30 |
| Group B | 0.13 | −0.05 | 0.29 | −0.01 | −0.05 | 0.13 |
| *t test* | *0.118* | *0.107* | *0.636* | *0.205* | *0.370* | *0.618* |
| **ΔCD4+ (cells/μL)** | | | | | | |
| Group A | −68.67 | −45.33 | −133.67 | −3.00 | 22.33 | 52.33 |
| Group B | −118.67 | −128.33 | −144.00 | −104.00 | −51.67 | −237.67 |
| *t test* | *0.564* | *0.435* | *0.958* | *0.430* | *0.361* | *0.156* |

*TRIM5* and *ZNRD1* genes was analysed, however their distribution did not varied in patients between group A and group B (Supplementary File 2).

Based on clustering, we decided to analyse differential gene expression separately in the two groups of donors, A and B. Within the 84 genes of the RT2 Profiler PCR Array there were at least three groups of modulated genes: genes with a p-value <0.05 and an absolute $\log_2$FC > 2 (significantly and highly modulated), genes with an absolute $\log_2$FC > 2 but not significantly different ($p > 0.05$) and genes with a p-value <0.05 but scarcely modulated ($\log_2$FC < 2) (Fig. 2). We decided to considered gene expression significantly different only when supported by a p-value <0.05 and an absolute $\log_2$FC > 2. In Supplementary Table 3 complete gene expression data are reported. Table 3 reports selected genes at all the differentiation's steps according to above-mentioned criteria. At some time points gene modulation did not reach a statistical significant p-value, however we have included these values (indicated with an asterisk) to emphasize that the direction of gene modulation did not vary along differentiation, neither after virus stimulation (4 h) nor after cytokines maturation cocktail (14 h, 24 h, 48 h).

In DC from A group only few genes appeared to be significantly and highly modulated (10 out of 84). The HIV natural ligands *CCL4* and *CD4,* the HIV induced transcription factor *IRF1* as well as the apoptotic genes *BAD* and *CASP8* resulted down-regulated compared to monocytes at all the considered time points (iDC, 4 h-DC, 14 h-DC, 24 h-DC and 48 h-DC). In a similar way, innate immune response genes, namely *IL1B, IL10, SELL, TNF* and *TNFSF10*, also appeared to be significantly down-regulated at least at one of the differentiation steps.

On the other hand, in cells from group B we observed a statistically significant modulation in 25 out of 84 genes in quite all the considered time points. In this group, 22 genes resulted up-regulated, whereas only 3 genes resulted down regulated (Table 3). Genes belonging to the apoptotic as well as to "cell activation by HIV-1" pathways were up-regulated in these DC compared to monocytes.

Only 5 genes seemed to be significantly modulated in both groups. *IL1B* and *SELL* were commonly down-regulated, whereas *BAD, CASP8* and *IRF1* were oppositely modulated, being down-regulated in DC from group A and up-regulated in group B (Table 3). This finding was not corroborated by viability assay. Mature dendritic cells (48 h-DC) obtained from all the patients were similarly viable (Group A: 80 ± 7% PI-negative cells; Group B: 75 ± 4% PI-negative cells; $p = 0.338$), however we cannot exclude that cell death could happen later than 48 h.

When looking at innate immune response genes (Fig. 3A), we can observe that the expression of *IL1B, IL10, SELL*, *TNF* and *TNFSF10* was significantly down-regulated in DC belonging to group A. However *IL1B* and *TNF* were less down-regulated in 14 h-DC respect to the other time points, maybe due to synergic effect of viral pulse and cytokines used for the culture. In group B, cells significantly modulated *FCAR, IL1B, IL12B, SELL* and *STAT1* (Fig. 3B). Of notice, expression of *IL12B* was augmented at the end of protocol, suggesting the ability of these DC to produce IL-12, a key cytokine in the context of immunologic synapsis.

The expression of transcription factors STAT1, IRF1 and NF-KB has been reported to contribute to the altered susceptibility to HIV infection [14], for this reason we considered and plotted the expression profile of those genes during DC manipulation (Fig. 3C and D), even if FC or p-value were out of our selection criteria. It is interest to notice that whether *NFKB* was weakly up-regulated in both groups, *STAT1,* and *IRF1* were down-regulated only in group A. As *IL12* gene is a target of IRF1, it is not surprising that only B group cells showed up-regulation of *IL12* (Fig. 3B).

Considering above-mentioned data about *IL1B* differential gene expression (Fig. 3), and the key role of this cytokine in DC biology as well as our previously reported data about the constitutive expression of inflammasome genes in DC from HIV$^+$ individuals [15], we evaluated the expression of inflammasome' genes *NLRP3, CASP1* and *IL18* in monocyte-to-DC differentiation with a gene specific probe assay. No significant difference in *NLRP3* or *CASP1* modulation was observed in DC compared to monocytes in the two groups (Fig. 4), compatible with *IL1B* expression data (Fig. 3) and in accordance with our previously published results [15]. Unexpectedly *IL18* was up-regulated in DC from group A donors, at 14 as well as at 48 h (2.38 and 1.94-fold, respectively), but not in group B cells (−0.29 and 0.42-fold, respectively). The difference between *IL18* expression in 48 h-DC of groups A and B resulted statistically significant ($p < 0.05$).

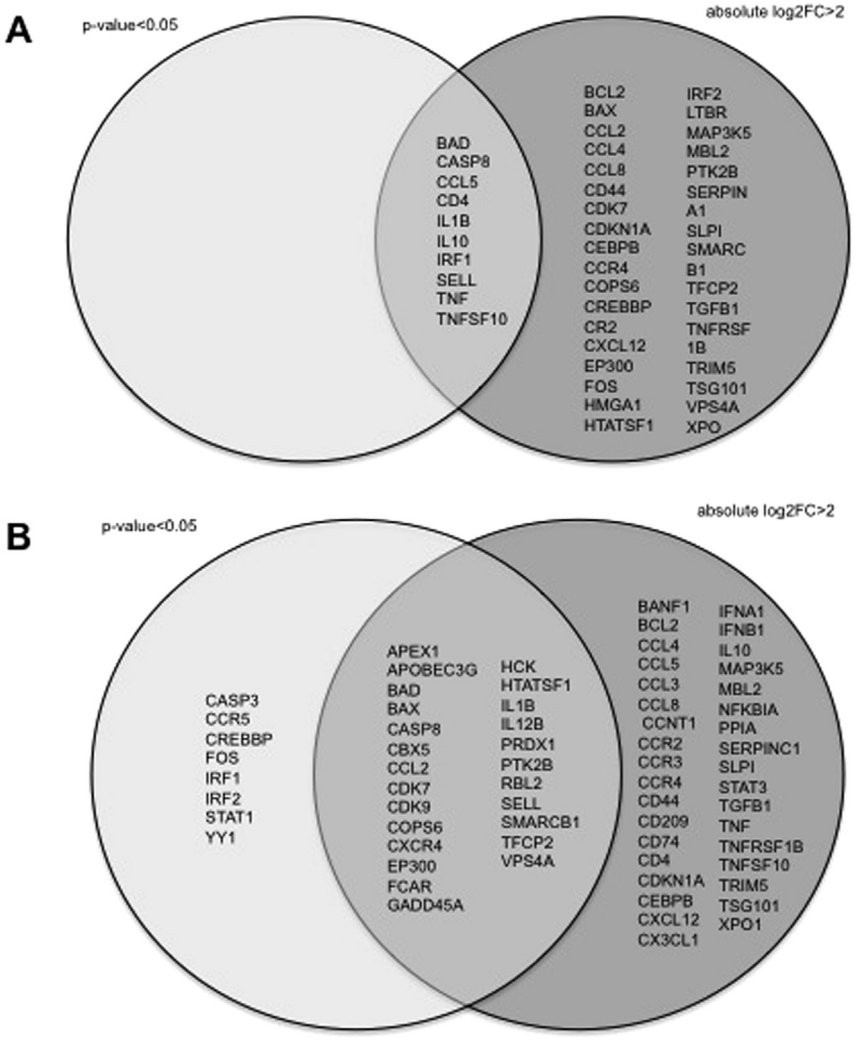


Fig. 2. **Selection of differential expressed genes**. Regulated genes in DC versus monocytes in group A **(A)** and B (**B**). Left circle represents differential expressed genes with a p-value <0.05; right circle represents differential expressed genes with a $log_2FC > 2$. Overlapping region represents genes with p-value<0.05 and $log_2FC > 2$. Circles are drowned in arbitrary scale.

Considering the difference of anti-HIV response genes expression between the two groups of patients, we wondered whether, before starting differentiation protocol, *ex vivo* peripheral blood monocytes just show a different expression profile. For this purpose, differential gene expression was evaluated compared group B versus group A monocytes, and only one gene, *CCR5,* resulted significantly up-regulated (16-fold, p = 1.99exp-4). This data suggests that monocytes belonging to group B could be more chronically activated [16] and this condition could affect also the chronic activation state of respective monocyte-derived dendritic cells, as we observed in B group DC (Table 3).

Then we investigated whether different molecular profile could affect dendritic cell characteristics or functionality. For this reason we analysed by cytometry cells surface markers in DC and monocytes as well as DC ability to activate autologous lymphocytes in co-culture assay.

HLA-DR resulted highly expressed in the surface of immature DC (iDC) as well as in mature DC (48 h-DC) in both groups; this is possibly due to the chronic infection as previously observed in monocytes-derived DC from $HIV^+$ [17]. As expected, maturation and activation markers - CD40, CD80, CD83 and CD86 – resulted significantly augmented in mature DC compared to iDC in both groups of cells ($p < 0.05$) (Fig. 5A). However, any significant difference in markers expression between A and B groups were observed ($p > 0.05$), suggesting that, at least from a phenotypic point of view, DC from all the donors are very similar.

When looking at surface markers in *ex vivo* monocytes, as the precursors of *in vitro* manipulated DC, monocytes from group B donors appeared to be more activated compared to those from group A (HLA-DR: 40% versus 57.6%; CD11c: 49.6% versus 98.2%; CD86: 48.5% versus 94.2%) (Fig. 5B), even if this difference did not achieve significant threshold ($p > 0.05$) possibly due to the limited size of samples and the well known PBMC inter-individual heterogeneity. Wondering whether B group patients could have all PBMC more activated, peripheral blood lymphocytes were analysed for surface expression of activation molecule CD38, however no differences have been observed (Fig. 5C).

Table 3
**Differentially expressed selected genes in groups A and B dendritic cells compared to monocytes.**

| Group A | | | | | | Group B | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Genes | iDC | 4 h | 14 h | 24 h | 48 h | Genes | iDC | 4 h | 14 h | 24 h | 48 h |
| **HIV receptors & natural ligands** | | | | | | | | | | | |
| *CCL5* | −6.86 | −6.45 | −1.25* | −3.34* | −3.36* | *CCL2* | NA | 2.42* | 1.82* | NA | −4.40 |
| *CD4* | −0.73* | 0.14* | −0.77* | −2.23 | −3.70 | *CXCR4* | 1.56* | NA | 2.62* | 3.17* | 6.05 |
| **Innate immune response** | | | | | | | | | | | |
| *IL1B* | −8.88 | −7.35* | −3.61* | −7.98* | −8.39* | *FCAR* | −9.97 | −9.30 | −10.31 | −12.83 | −16.89* |
| *IL10* | −4.74* | −3.70* | −4.07* | −5.86* | −6.11 | *IL1B* | −2.61* | −2.62* | NA | −1.51* | −5.94 |
| *SELL* | −4.63* | −5.12* | −5.30 | −6.59* | NA | *IL12B* | −4.18* | −6.23 | NA | 2.07* | 2.02* |
| *TNFSF10* | −7.63 | −7.80* | −7.00* | −10.51* | NA | *PRDX1* | 2.79 | 2.44* | 2.68 | 2.99 | 3.33 |
| *TNF* | −8.59 | −7.56* | −2.50* | −6.44* | −6.21* | *SELL* | NA | −2.07* | −1.82* | −1.73* | −4.32 |
| **Cellular proteins induced or activated by HIV infection** | | | | | | | | | | | |
| ***BAD*** | −12.45 | −11.98 | −6.03 | −11.97 | −10.98 | ***BAD*** | 4.28 | 4.06 | 4.01 | 4.13 | 2.74* |
| ***CASP8*** | −3.23* | −2.17* | −1.70 | −3.89 | −4.63* | *BAX* | 1.20* | NA | NA | 1.02* | 5.62 |
| *IRF1* | −7.06 | −5.43* | −2.87* | −5.04* | −4.11* | ***CASP8*** | 2.24 | 2.27 | 1.86 | 2.35 | 1.02* |
| | | | | | | *COPS6* | 2.19 | 2.15 | 1.96 | 2.04 | 4.41 |
| | | | | | | *GADD45A* | 2.82 | 2.75* | 3.60 | 4.53 | 6.21 |
| **Cellular cofactors involved in HIV infection** | | | | | | | | | | | |
| | | | | | | *APEX1* | 3.51 | 3.32 | 3.41 | 3.16 | 5.37 |
| | | | | | | *APOBEC3G* | 3.11 | 2.49* | 2.36* | 3.20 | 4.68 |
| | | | | | | *CBX5* | 1.62* | 1.66* | 1.71* | 1.28* | 5.54 |
| | | | | | | *CDK7* | 2.49* | 2.34* | 2.15* | 2.59* | 4.98 |
| | | | | | | *CDK9* | 1.45 | NA | 1.37 | 1.37 | 3.77 |
| | | | | | | *EP300* | 2.69* | 2.63* | 2.28 | 2.35* | 4.86 |
| | | | | | | *HCK* | 2.93 | 2.55 | 2.87 | 3.15 | 5.66 |
| | | | | | | *HTATSF1* | 2.90 | 2.94 | 2.50 | 2.77 | 4.90 |
| | | | | | | *PTK2B* | 2.58 | 2.21 | 1.58* | 2.29* | 4.57 |
| | | | | | | *RBL2* | 2.31 | 2.18 | 1.69 | 2.14 | NA |
| | | | | | | *SMARCB1* | 2.33 | 2.29 | 2.13 | 2.26 | 2.71 |
| | | | | | | *TFCP2* | 3.15 | 3.03* | 2.36* | 3.08* | 3.50 |
| | | | | | | *VPS4A* | 2.15 | 2.17 | 2.04 | 2.01 | 2.87 |

To determine the capacity of DC from groups A and B donors to activate *in vitro* autologous lymphocytes, CD38 surface expression and intracellular IFN-γ were measured in lymphocytes from co-culture assays. CD38 expression was augmented in B group $CD3^+$ T cells compared to A group (21.8% versus 12.3%), however also in this case the difference was not statistically significant ($p > 0.05$) (Fig. 6A). Intracellular IFN-γ staining revealed that DC from both groups were similarly able to induce IFN-γ production in $CD3^+$ T cells (Fig. 6B).

Finally, to evaluate any *in vivo* effect of studied DC, we compared difference (Δ) in PVL, $CD4^+$ and $CD8^+$ counts after immunotherapy in individuals of groups A and B. Clinical data have been collected before each of the three immunotherapy doses (t1, t2, t3) and after 1, 2 and 6 months from the third dose (t4, t5, t6). PVL did not diminished during treatment. Apparently group A DC induced a better $CD4^+$ and $CD8^+$ ($CD3^+$) increment compared to group B DC (Supplementary Table 3), however this difference was not statistically significant at any of the time points.

## 4. Discussion

Liu et coll [16] have demonstrated that DC gene expression profile could be used as a predictor of function and help the design and/or patients selection of DC-vaccine trials in cancer therapy. With a similar purpose the differential expression of a subset of genes involved in host anti-HIV response was analysed in dendritic cells used in the on-going Brazilian clinical trial of anti-HIV immunotherapy.

Gene expression analysis revealed a distinct profile in monocyte-to-DC differentiation within $HIV^+$ individuals submitted to immunotherapy. This profile did not apparently correlate with initial clinical data such as $CD4^+$ and $CD8^+$ cells count or plasma viral load (Table 1), nor with well-known genetic factor involved in HIV infection pathogenesis, suggesting the need to investigate novel characterization markers for DC in the contest of immunotherapy.

Cells from group B appeared to be chronically activated in term of HIV-response, showing an up-regulation of both restriction and co-factors for HIV-1, but this augmented expression did not significantly vary along differentiation, possibly being an intrinsic characteristic of these patients (genetic background, chronic inflammation state) and not a consequence of dendritic cell preparation protocol. Moreover these cells seemed to be more prone to programmed cell death than group A, as several pro-apoptotic genes (i.e.: *BAD, BAX, CASP8*) were significantly up-regulated in group B. On the contrary, *BAD* and *CASP8* resulted down-regulated in DC of group A. Giri et coll [7], showed that monocytes from $HIV^+$ individuals are characterized by an anti-apoptotic

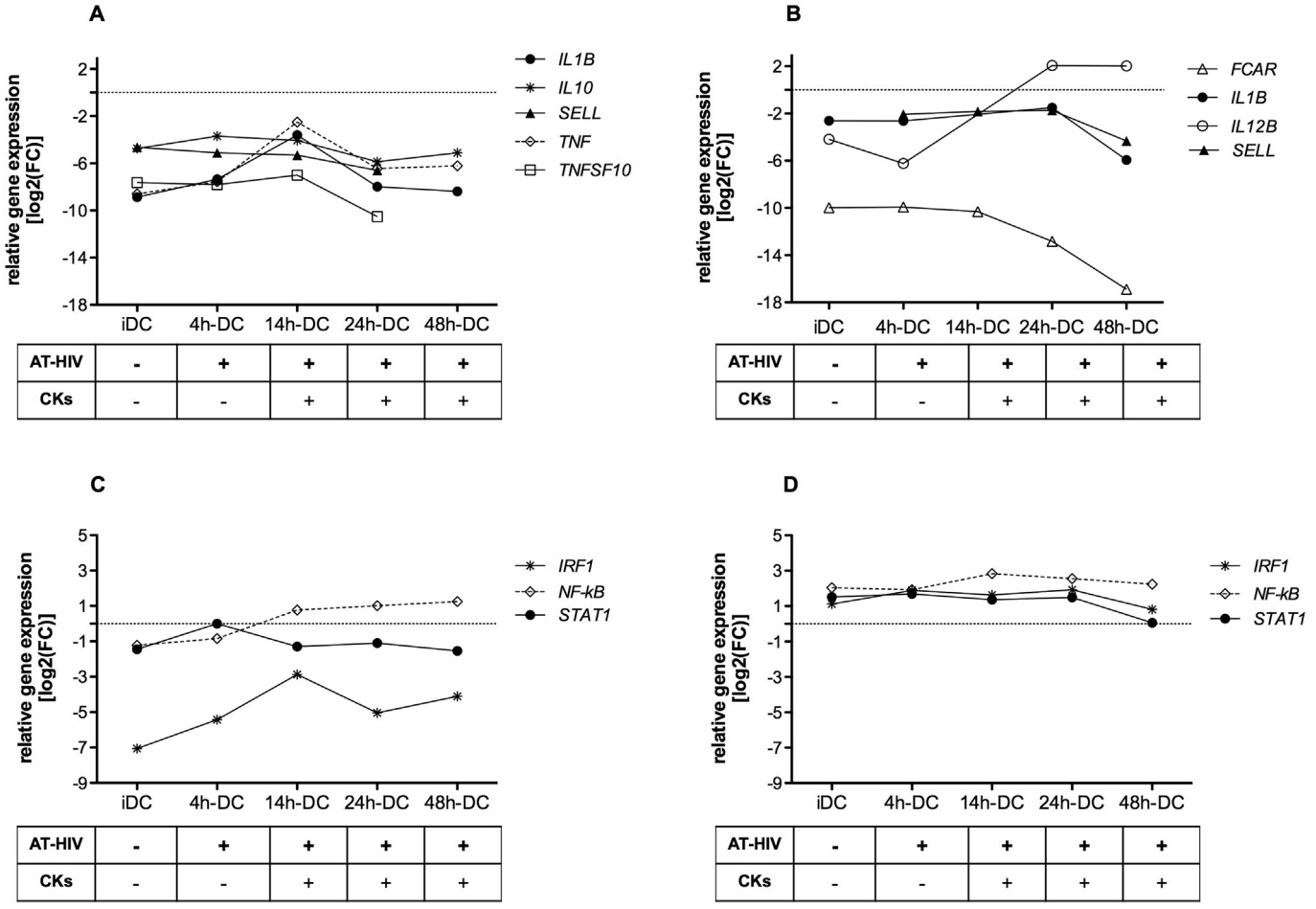


Fig. 3. **Expression of innate immune genes during DC differentiation**. Relative gene expression of selected innate immune genes in groups A and B dendritic cells is reported during differentiation steps (iDC, 4 h-DC, 14 h-DC, 24 h-DC and 48 h-DC) compared to monocytes. *IL1B, IL10, SELL, TNF* and *TNFSF10* are reported for A group DC (**A**); *FCAR, IL1B, IL12B, SELL* for B group DC (**B**); *IRF1, NF-kB* and *STAT1* are reported for A group DC (**C**) and B group DC (**D**).

signature, however, to our knowledge, no data have been reported about monocyte-derived DC. We can hypothesize that monocytes-to DC differentiation protocol may act in different way according to original monocytes expression profile, suggesting that activation state of monocytes could be taken into account as a early predictor of DC characteristics before mature DC viability result, that nowadays represents one of the main quality control data for DC application in patients.

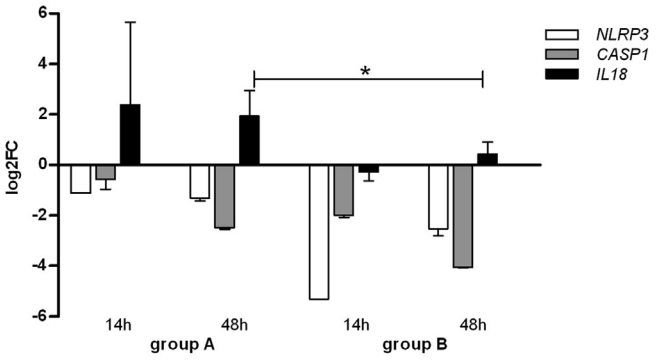


Fig. 4. **Expression of inflammasome genes during DC differentiation**. Relative gene expression of *NLRP3, CASP1* and *IL18* genes in groups A and B dendritic cells is reported at 14 h-DC and 48 h-DC compared to monocytes. One-way ANOVA test was used to compare A and B groups. *=<0.05.

*IRF1* expression had previously been described as a factor that contributes to susceptibility for HIV-1 infection [14]. Our findings evidenced a different expression modulation of this transcription factor in the two groups of DC according with the expression of the important Th1 driving cytokine IL-12 (Fig. 2), emphasizing once more that genomic profile could told us a hide tale about DC functionality.

The emerging role of IL-18 in the pathogenesis of HIV infection has been recently described, suggesting that IL-18 could be protective against HIV replication [17]. IL-18 plays an important role in DC biology, being necessary for induction of effector T cells [18] and memory $CD8^+$ T cells [19]. Moreover it has been reported that in DC augmented level of IL-18 inversely correlated with IL-10 [20], as observed also in our results (Table 3).

In our study, possibly due to the small size of studied individuals, and we are aware of this limitation, the observed differences in genes expression did not lead to statistically significant differences in commonly used markers of DC maturation, activation and *in vitro* ability to induced IFN-γ+ T cells.

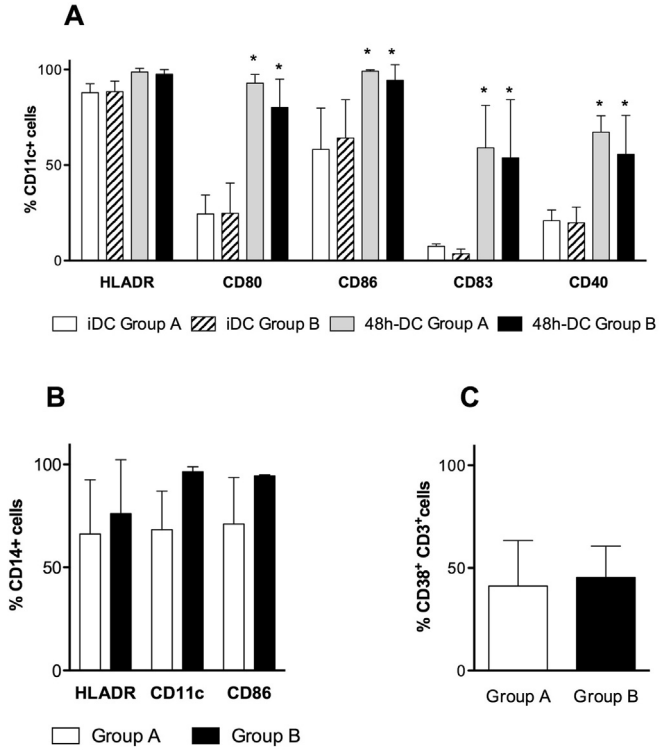


Fig. 5. **Surface activation markers analysis**. (**A**) Phenotypic profile of immature DC (iDC) and mature DC (48 h-DC) of A and B groups. Mean values and standard errors (n = 3) are reported. DC were analysed according to basic characteristics of size and granulosity (not shown) and specific DC markers (CD11c, HLADR, CD80, CD86, CD83, CD40). Two-ways ANOVA test was used to compare iDC and 48 h-DC within each group and between A and B groups. *=<0.05 refers to 48 h-DC versus iDC. (**B**) Phenotypic profile of *ex vivo* monocytes of A and B groups. Mean values and standard errors (n = 3) are reported. Monocytes were analysed according to basic characteristics of size and granulosity (not shown) and specific markers (CD14, CD11c, HLADR, CD86). One-way ANOVA test was used to compare A and B groups. (**C**) Phenotypic profile of post-adherence lymphocytes of A and B groups. Mean values and standard errors (n = 3). Lymphocytes were analysed according to basic characteristics of size and granulosity (not shown) and specific markers (CD3, CD38). T-test was used to compare A and B groups.

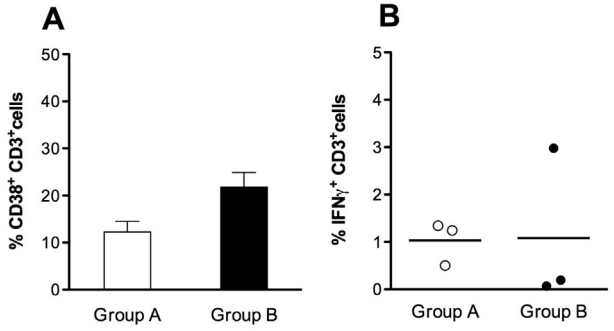


Fig. 6. **DC-mediated activation of autologous lymphocytes.** (**A**) Phenotypic profile of post-co-culture lymphocytes of A and B groups. Mean values and standard errors (n = 3) are reported. Lymphocytes were analysed according to basic characteristics of size and granulosity (not shown) and specific markers (CD3, CD38). T-test was used to compare A and B groups. (**B**) IFN-γ production in $CD3^+$ T cells in A and B groups. Mean values (n = 3) are reported. Lymphocytes were analysed according to basic characteristics of size and granulosity (not shown), surface marker CD3 and intracellular staining IFN-γ. T-test was used to compare A and B groups.

## 5. Conclusions

All together these findings pointed out that actual criteria for the selection of $HIV^+$ individuals for immunotherapy (mainly PVL, $CD4^+$ and $CD8^+$ counts) are not ensuring a similar vaccine product in term of genomic activation of monocyte-derived DC. Further investigations are needed to elucidate the discrepancy between expression profiles in DC from different donors.

Clinical trials generally are not designed for genetic approaches and, especially for immunotherapy, which is highly time- and money-consuming, the number of enrolled patients always would represent a limit. This study belongs to a larger research work aimed to explore genetic background of immunotherapy response and to identify predictive marker for treatment success. We are convinced that response to DC-based vaccine has to be considered as a multifactorial tract, where genetic factors should be taken in account in the choice of patients as well as in DC preparation design.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

AP designed and coordinated the study, analysed and interpreted the data, and draft the manuscript; ECR carried out the gene expression experiments and dendritic cells assays; LTS carried out virus isolation and dendritic cells culture; AJSD is the coordinator of the on-going immunotherapy clinical trial; SC contributed to design of the study, interpretation of data, and to draft the manuscript; TMO coordinated dendritic cells production and contributed to draft the manuscript.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jocit.2016.03.002.

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